

## Molecular Diagnosis on Glucose 6 Phosphate Dehydrogenase in a Sample of Iraqi Patients

Rehab S. Ramadhan<sup>1</sup>, Hadeel M. Khalaf<sup>1</sup> and Rebah N. Jabber<sup>2</sup>

<sup>1</sup>Department of Medical Biotechnology, College of Biotechnology,  
Al-Nahrain University, Baghdad, Iraq.

<sup>2</sup>Biotechnology Research Center, Al-Nahrain University, Baghdad, Iraq.

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This study was constructed to investigate Glucose 6 phosphate dehydrogenase deficiency, and the genetic disorder that leading to hemolysis anemia in group of Iraqi patient. A total of 50 blood samples were collected from different hospitals (Yarmook Hospital, Child hospital, AL Alweyaa, and Medical City) these samples varied in severity in of the enzyme deficiency from mild to chronic according to the test of G6PD, and 20 samples served as control (healthy). The period time of samples collection took about three months from March to June 2014. The percentage of mild symptoms was 42%, acute symptoms was 40% and carrier G6PD was 18% as stated on statistics analysis of Chi square. DNA was extracted from samples and subjected to PCR amplification using 3 specific primers designed for purpose of this study, the first primer (Frag I) with product length 115 bp, second primer (FragII) with product length 1500 bp, third primer (Frag III) with product length 2000 bp that amplify the specific site for the carrier and mild patients with G6PD, while no band was amplified for the chronic patients with G6PD. After comparing cases and with NCBI we found that the percentage insertion was 30% and substituted was 70%.

**Key words:** G6PD, type mutation of gene, hemolysis anemia, and molecular diagnosis.

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Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common human enzyme defect, being present in more than 400 million people worldwide (Cappellini and Fiorelli, 2008). During glucose 6 phosphate dehydrogenase deficiency, the red cells are unable to regenerate reduced nicotinamide adenine dinucleotide phosphate (NADPH), a reaction that is normally catalyzed by the G6PD enzyme (Ademowo and Falusi, 2002). Since the X chromosome carries the gene for G6PD enzyme,

this deficiency mostly affects males. The two major conditions associated with G6PD deficiency are hemolytic anemias and neonatal jaundice, which may result in neurological complications and death (Luzzatto and Gordon-Smith, 2001). Screening and detection of G6PD deficiency helps in reducing such episodes, through appropriate selection of treatment, patient counseling, and abstinence from disease-precipitating drugs such as antimalarials and other agents. G6PD is an enzyme present in the cytoplasm of all cells, acting specifically in the maintenance of the integrity of the erythrocytes, preventing the oxidation of hemoglobin and other cellular proteins so the deficiency of G6PD will cause hemolytic anemia and jaundice induced by ingestion of oxidative drugs and/or broad beans, there are different kinds of hemolysis from mild to

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\* To whom all correspondence should be addressed.  
E-mail: rebahalgafari@gmail.com

severe that are seen to differences in variants of the disease (Au *et al.*, 2006). Molecular studies have shown that the G6PD deficiencies are nearly always caused by single amino acid substitutions (Poggi *et al.*, 1990).

## MATERIALS AND METHODS

Blood Samples were collected from 50 persons suffering from G6PD and, 20 healthy as a control (healthy), their ages ranged between (7 day – 20 years). Samples were subjected to centrifugation at 2000 rpm for 10 min, and was used for DNA extraction. The Samples were obtain from Yarmook Hospital, Child hospital, AL Alweyaa, and Medical City. The collection period from March 2014 to June 2014.

### Detection in Glucose 6 phosphate dehydrogenase laboratory

The detection of G6PD was performed using colorimetric kit from Sigma USA according to manufacturer instructions.

### DNA Extraction

Total cellular DNA was extracted from blood samples by using the Reliaprep Blood genomic DNA MiniPrep System from Favorgene Taiwan, determination of concatenation and purity of the extracted DNA was done using nanodrop (Techne /UK).

### PCR Protocols

Extracted DNA from blood samples and healthy was used in PCR for amplification of Frag I, Frag II, Frag III primers table (1) Initial denaturation 94°C for 5 min., 35 cycle contain denaturation 94°C for 1 min, Annealing 60°C, 61°C, and 59°C for each gene, extension 72°C for 1 min., and final extension 72°C for 10 min.

### DNA sequencing

Polymerase chain reaction products of G6PD gene using Frag I, II and III for 30 blood

samples were sequenced, and aligned with NCBI to examine the presence of SNPs.

### Statistical Analysis

The statistical Package for the social science (SPSS, version 14) was used for statistical analysis of Chi-square and deviation from Hardy Weinberg equilibrium distribution of different groups between patients and control, p value < 0.01 was considered statistically significant.

## RESULT AND DISCUSSION

### The distribution of the studied group

Glucose -6-phosphate dehydrogenase (G6PD) deficiency is the common disease. More than 300 variants of G6PD characterized by standard method (Farhud, and Yazdanpanah, 2008).

In this study, fifty sample were collected from patient diagnosed with G6PD (males 37, females 13) as shown in table (2).

### Molecular detection of G6PD deficiency by PCR technique

Three primer sets were designed using the NCBI Primer-Design online tool. In order to amplify a specific region in the G6PD gene by using a routine PCR technique.

The G6PD enzyme exists in its active form, as a dimer (or tetramer) each of which consist of 515 amino acid polypeptide subunits (Mason *et al.*, 2007). Each dimer contain tightly bound NADP that plays both structural and functional roles. The enzyme is encoded by G6PD gene, is located on the long arm of the X-chromosome (Xq28) and spans over 18 kilobases consisting of 13 exons and 12 intron (non-coding sequences) (Hesham *et al.*, 2011). The most common mutation occur in exon 7 and frequently detected in Mediterranean, Middle Eastern Hirono and Beutler (1988)

The first primer set used in this PCR technique (Frag I) specific for the intron (6) of G6PD

**Table 1. Primers**

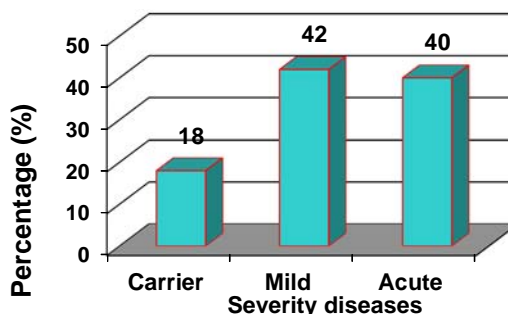
Name	Sequence (5'-3')	Product length	References
Frag I	F:CTGAAATCTGGCCTCTGTCC R:GTTTCAGCCCCATCTTAGCAG	115 bp	NCBI
Frag II	F:ACCACAAGGTGGCAGCGTTG R:TGCCTTGCTGGCCTCGAAGG	1600 bp	NCBI
Frag III	F:CCAGGGACGTGATGCAGAAC R:GGGCAGGGACATGGACAGTAAGAG	2000 bp	NCBI

**Table 2.** Description of sample study according to gender

Gender	Number	Percentage (%)
Male	37	74.00
Female	13	26.00
Total	50	100%
Chi-square value	-	11.826 **

\*\* (P<0.01).

50 samples divided into three groups depending on the severity of the disease as show in figure (1)



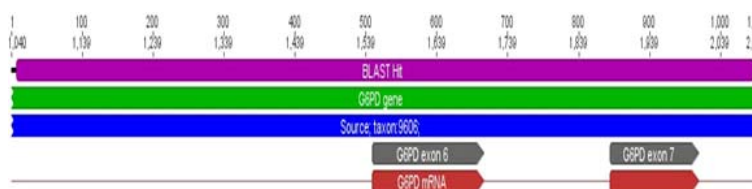
**Fig. 2.** The distribution of patient depending on the severity disease



**Fig. 4.** Ethidium bromide-stained PCR products after gel electrophoresis. Primer set was used to amplify a sequence of G6PD gene. (2% agarose gel, 10 minutes at 100 voltage and then lowered to 70 Volts, 50 minutes). Negative result was noticed in sample 4,6,10, positive samples were 1,2,3,5,7,8,9 thus indicate successful amplification of the target sequence. The gel also shows a positive control (C), negative control (N), and M represent DNA ladder.



**Fig. 5.** Ethidium bromide-stained PCR products after gel electrophoresis. Primer set was used to amplify a sequence of G6PD gene. (1.5% agarose gel, 10 minutes at 100 voltage and then lowered to 70 Volts, 1 h and 30 minutes) Negative result was noticed sample 1,2,3,4,5,7,10. Positive sample were 6,8,9, thus indicate successful amplification of the target sequence. The gel also shows a positive control (C), negative control (N), and M represent DNA ladder.



**Fig. 6(a).** Sequence identity of G6PD gene, the amplify segment include exon 6 and 7.



**Fig. 6(b).** Display point mutations occur in the amplified region in G6PD gene

gene from NCBI primer design with product length (115 bp) which is shown in the figure (1)

The result of this amplification revealed that (70%) were positive and this percentage similar to (Jalloh *et al.* 2004).

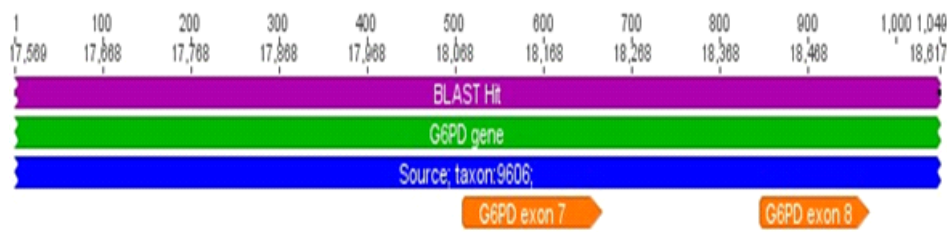
The second primer set used in PCR technique (Frag II) was special to amplify exon 6 and 7 of G6PD gene with product length 1500 bp shown in figure (5).

Four bands were observed in the gel with size nearly 1500 bp. (Ademowo *et al.*, 2002)

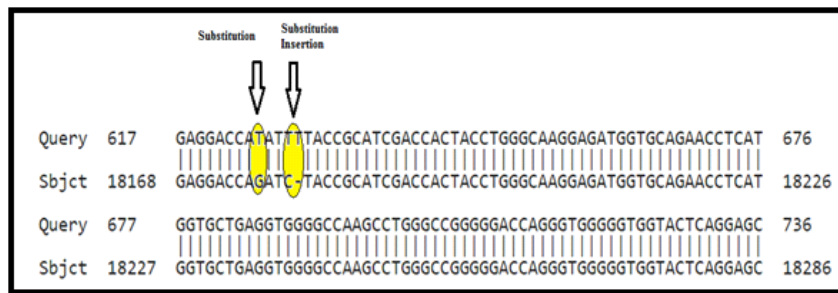
The sequencing of the G6PD gene from

the all patients in Iraqi populations using primer Frag II and located in exons 6,7 and 8.

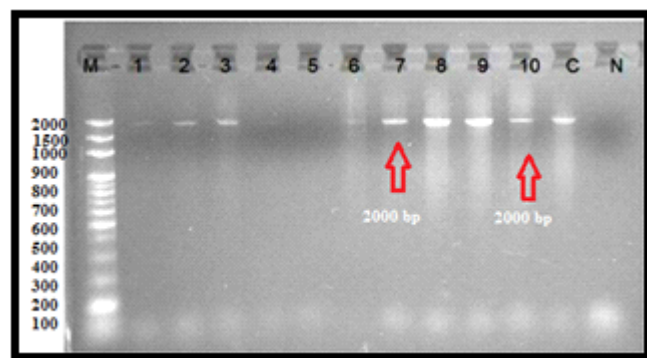
The amplified sequence in the current study is illustrated in chromosome X, taxon: 9606 in G6PD gene. The segment length was exactly 1049 bp with the upper line (Blast hit) and the sequence amplified started from 1047 – 2095 in the origion NCBI gene include exon 6 with length 159 bp (1548 - 1706) and exon 7 with length 126 bp (1884 – 2009).The green line represent the NCBI control.



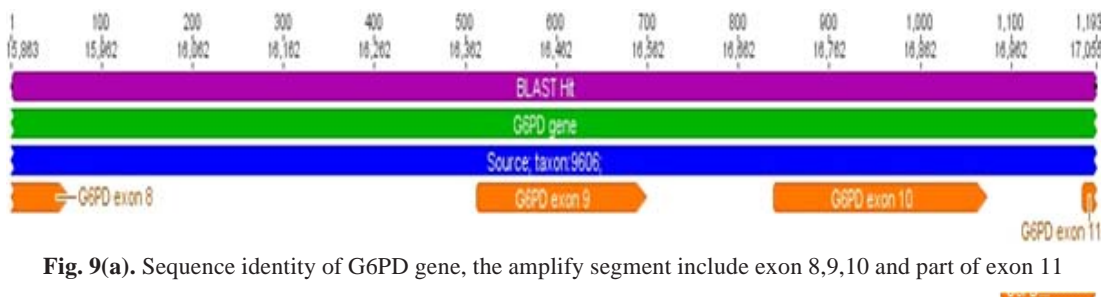
**Fig. 7(a).** Sequence identity of G6PD gene, the amplify segment include exon 7 and 8.



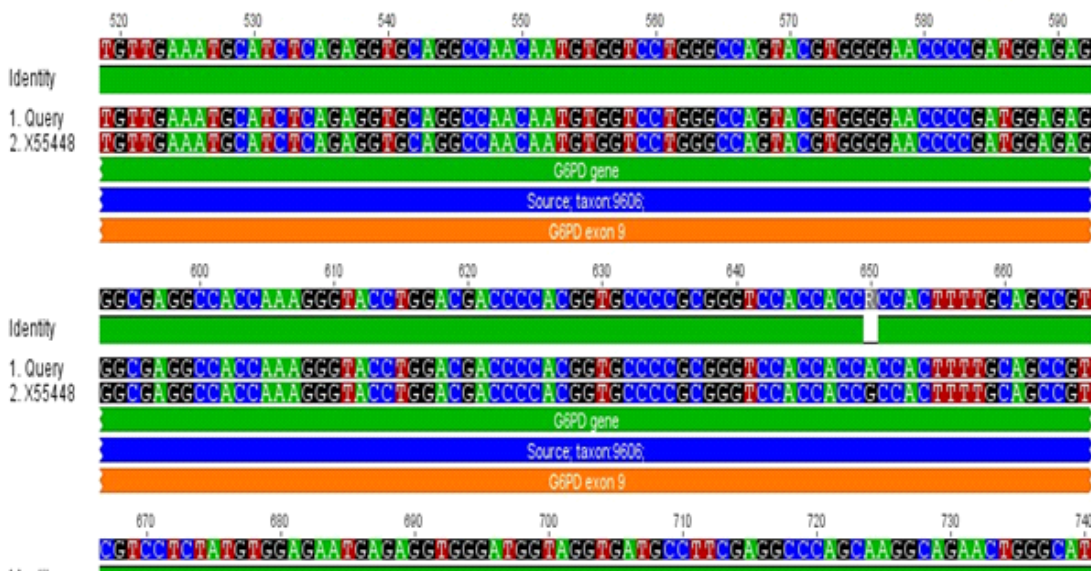
**Fig. 7(b).** Display point mutations occur in the amplified gene in exon 7 after comparing with NCBI blast



**Fig. 8.** Ethidium bromide-stained PCR products after gel electrophoresis. Primer set was used to amplify a sequence of G6PD gene. (1.5% agarose gel, 10 minutes at 100 voltage and then lowered to 70 Volts, 2h) negative result was noticed in sample 5. Positive samples were 1,2,3,4,6,7,8,9,10,thus indicate successful amplification of the target sequence. The gel also shows a positive control (C), negative control (N), and M represent DNA ladder



**Fig. 9(a).** Sequence identity of G6PD gene, the amplify segment include exon 8,9,10 and part of exon 11



**Fig. 9(b).** Display point mutations occur in the amplified region in exon 9 after comparing with NCBI blast

Point mutation occurred in the amplified region include transition mutation conversion C/T at position 616, beside to other mutation T insertion occur at position 617. (Vulliamy *et al.* 1988) and the same change C / T change at position 563 in exon 6 cause the conversion Ser / Phe at position 188 such conversion might cause loss of enzyme function and the ability to react normally, other mutation was noticed by (Taki *et al.* 2001) when A/G in exon 6 cause the conversion Asn / Asp was also observed.

In G6PD gene the segment length was amplified exactly 1258 bp start from 18077 - 18235 in the original NCBI gene include exon 7 with length 159 bp and exon 8 with length 126 bp.

Three point mutations occur in the amplified segment in exon 7, the first was a transversion mutation convert G/T at position 625, second was a transition mutation C/T at position 628, the other mutation was insertion of T at

position 629. This variant called the Mediterranean variant, and it has higher affinity for G6PD and reduced thermo stability and associated with acute hemolytic anemia in response to therapeutic drugs and fava beans (Cappellini, *et al.* 2008).

The third primer set used in PCR technique (Frag III) exclusively for the exon (9) with product length more than 1000 bp as shown in figure (3-7).

Through four exons length of the amplified segment was exactly 1193 bp start from 15863 - 17055 in the original NCBI gene include part of exon 8 and complete exon 9 with length 187 bp, exon 10 with length 236 bp start from 16701-16936 at the original fragment and part of exon 11 in G6PD gene.

Point transversion mutation occur when G convert to A at position 650 in exon 9 in G6PD gene. Moradkhani *et al.* (2012) determine a point mutation in exon (9) of G6PD gene at 1003 when G



(guanine) convert to A (adenine) cause change in amino acid Arg to gln

Nine positive amplification products were observed the gel with amplified size more than 1000 bp. This result was similar to (Zhao *et al.*, 2004)

The overall look for this change might explain the loss of activity or change in stability or in some cases leading to severe enzyme deficiency, enzyme deficiency mostly caused by a point mutation in exon 7 that encoded the most important amino acids for enzyme stability, (Au *et al.*, 2000).

Analysis of G6PD gene by sequencing for Iraqi patients exhibited the existence of many genetic alteration. Two types of mutations were found substitution, and insertion in 70% and 30% respectively. Xu *et al.* (2005) found the percentage of substitution point mutation in G6PD gene was more than insertion about 70%.

Most of the genetic variants tend to be single-point mutations, and the lack of large or out-of-frame deletions may indicate that the total absence of enzyme activity is fatal. Insertion were usually associated with the most severe clinical manifestations (class I), Mason *et al.* (2007).

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